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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Serial No. 10/660,301

Customer No. 23379

Applicant: Giroir et al.

Confirmation No. 5400

Filed: Sep 10, 2003

Group Art Unit: 1644

Docket No. UTSD:1477

Examiner: Chun Crowder

Title: *Macrophage Migration Inhibitory Factor as a Marker for Cardiovascular Risk*

DECLARATION UNDER 37CFR1.131

1. We are the coinventors of the subject application

2. Attached Exhibit A is a copy of a draft patent application that patent attorney Richard Osman sent to the undersigned inventors on Aug 19, 2003.

4. Between Aug 19, 2003, and the Sep 10, 2003 filing date of the subject application we were diligent in reviewing and preparing the application for filing.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application and any patent issuing therefrom.

Date: 1/2/07 _____

Brett P. Giroir, M.D.

Date: _____

Monte S. Willis, M.D., Ph.D.

Date: _____

Timothy S. Church, M.D., M.P.H., Ph.D.

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DECLARATION UNDER 37CFR1.132

I, Dr. Brett Paul Giroir declare and state as follows:

I am the Director of the Defense Sciences Office (DSO) of the Defense Advanced Research Projects Agency (DARPA). I received my undergraduate degree from Harvard University (magna cum laude, biology, 1982), and my medical degree from the University of Texas Southwestern Medical Center (AOA, 1986). I am board certified in pediatric critical care medicine and am a previous member of the American Board of Pediatrics. Prior to my current position at DARPA, I was Professor and Associate Dean for Clinical Affairs at UT Southwestern Medical Center. In addition, I served as Physician-In-Chief and Chief of Critical Care Medicine and Trauma Critical Care at Children's Medical Center of Dallas. The Board of Regents of the University of Texas System is a co-assignee of this patent application. I have authored numerous scientific papers in the field of medical diagnostics and treatment, including several papers pertaining to cardiovascular medicine. I am a coinventor of this patent application.

2. What is "a person without cardiovascular disease or without a diagnosis thereof" is self-evident to one skilled in the art, and the use of the phrase in the Specification (e.g. p.3, lines 8-13) and Claims is consistent with how one skilled in the art would understand this term. One of ordinary skill in the art (e.g. a cardiovascular health professional) would understand the metes and bounds of this phrase.

What is an "apparently healthy individual" as recited in the preamble of claim 7 is self-evident to one skilled in the art, and the use of the phrase in the Specification (e.g. p.3, lines 9-13) and Claims is consistent with how one skilled in the art would understand this term. One of ordinary skill in the art (e.g. a cardiovascular health professional) would understand the metes and bounds of this phrase.

What is meant by the phrase "characterizing the individual's risk of developing the cardiovascular disorder based upon the combination of the first risk value and the second risk value, wherein the combination of the first risk value and second risk value establishes a third risk value different from said first and second risk values" is evident to one skilled in the art in view of the Specification (e.g. p. p. 3, lines 1-5 and p. 6, lines 13-15). One of ordinary skill in the art (e.g. a cardiovascular health professional) would understand the metes and bounds of this phrase.

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In my opinion, the claims are sufficiently clear such that one of ordinary skill in the art would understand the metes and bounds of the claims and be on notice as to what is the scope of the claims.

3. The invention of claim 1 comprises a two-step method of determining cardiovascular risk in a person without cardiovascular disease or without a diagnosis thereof: (i) determining a test MIF concentration in the blood, saliva or urine of the person as a marker of cardiovascular risk for the person, wherein an elevated test MIF concentration compared with a control MIF concentration not associated with cardiovascular risk indicates that the person is subject to elevated cardiovascular risk, and (b) a further step selected from the group consisting of: (a) assigning to the person a cardiovascular risk metric in accordance with the test MIF concentration; (b) prescribing for the person a cardiovascular treatment modality in accordance with the test MIF concentration; and (c) making an additional assessment of cardiovascular risk of the person in accordance with the test MIF concentration, the additional assessment selected from the group consisting of a stress test, a CRP assay and an LDL assay. The Specification plainly enables this two-step method:

(i) As taught in the Specification, MIF levels can be determined by a variety of art recognized methods:

> Typically, the level is determined by measuring the level of the marker in body fluid, such as blood, saliva or urine. The level can be determined by immunoassay or other techniques for determining the presence of the marker. A commercial human MIF ELISA detection kit is available from Chemicon (Temecula, Calif.), now Serologicals Corp. (Atlanta, Ga.). Automated analyzers on which tests for MIF can be performed include Dade Behring BN II Plasma Protein System (Dade Behring, Incorporated, Deerfield, Ill., USA), Abbott Laboratories IMx Immunoassay Analyzer (Abbott Laboratories, Abbott Park, Ill., USA), IMMULITE (Diagnostics Products Corporation, Los Angeles, Calif., USA), and IMMAGE (Beckman Coulter, Inc., Fullerton, Calif., USA). The Dade Behring BN II assay utilizes a monoclonal antibody on a polystyrene particle with fixed-time nephelometric measurements. The Abbott IMx assay is a two-site chemiluminescent enzyme immunometric assay with one monoclonal and one polyclonal anti-MIF antibody. The Beckman Coulter IMMAGE assay uses a polyclonal anti-MIF antibody on latex particles with rate nephelometric measurements. Specification p. 3, line 29 – p. 4, line 10.

The Specification plainly enables one skilled in the art to practice this step without undue experimentation.

(ii) (a) Taken in context, the claims recite "assigning to the person a cardiovascular risk metric *in accordance with his/her MIF concentration*". This step requires no more than assigning to the person a metric proportional to his/her MIF concentration (e.g. Specification, p.2, lines 16-17). The particular form of metric used is discretionary to the practitioner; e.g. numerical metrics such as "risk level 1, risk level 2, etc" or more descriptive metrics such as "very high risk, high risk, normal risk, low risk, etc. In view of the Specification, one skilled in the art is well-enabled to practice this step without undue experimentation.

(ii) (b) Prescribing a person a cardiovascular treatment modality in accordance with the person's risk of cardiovascular disease is routine in the art; for example, a person having an elevated MIF concentration may be treated with anti-inflammatory therapies; e.g. Specification p. 4, lines 22-25.

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(ii) (c) The recited stress test, CRP assay, and LDL assay are also well-known and routine to those skilled the art. In view of the Specification, one skilled in the art is well-enabled to practice this step without undue experimentation.

The Specification enables one skilled in the art to practice the two-step method of Claim 1 without undue experimentation.

The invention of Claim 7 comprises a three-step method for characterizing a risk of developing a future cardiovascular disorder in an apparently healthy individual. The recited obtaining and comparing steps use the same methodology as the determining step of Claim 1, and are demonstrably readily practiced by one skilled in art without undue experimentation (supra). The third recited step, characterizing an individual's risk of developing a cardiovascular disorder based on his/her level of a cardiovascular disease marker (MIF) is similarly readily practiced without undue experimentation. This step requires no more than assigning to the person a metric proportional to his/her MIF concentration (e.g. Specification, p.2, lines 16-17). The particular form of metric used is discretionary to the practitioner; e.g. numerical metrics such as "risk level 1, risk level 2, etc" or more descriptive metrics such as "very high risk, high risk, normal risk, low risk, etc." In view of the Specification, one skilled in the art is well-enabled to practice this step without undue experimentation. The Specification provides the requisite teaching that elevated levels of MIF in apparently healthy persons are predictive of future cardiovascular disorders (e.g. p. 2, lines 26-28).

The invention of Claim 15 is a two-step method for evaluating the likelihood that an individual will benefit from treatment with an agent for reducing the risk of a cardiovascular disorder. The recited obtaining and comparing steps use the same methodology as the determining step of Claim 1, and are demonstrably readily practiced by one skilled in art without undue experimentation (supra). The Specification provides the requisite teaching that MIF is elevated in patients with high cardiovascular risk, and that it falls when interventions are made which reduce the risk (e.g. Specification, p. 1, lines 29-31), i.e. that the test MIF level is indicative of whether the individual will benefit from treatment with the agent.

In my opinion, one of ordinary skill in the art would be able to practice the claimed invention without undue experimentation.

4. The invention is a method of determining cardiovascular risk in a person not predetermined to be subject to cardiovascular disease by determining the MIF concentration in the blood, saliva or urine of the person as a marker of cardiovascular risk for the person. The concept of a "marker of cardiovascular risk" implies to one skilled in the art that the marker is different in the risk group and in a corresponding control group. Furthermore, what you call the measure from the examined person ("test", "subject", etc.) and what you call the compared-to measure ("control", "predetermined value", etc.) are arbitrary and self-evident, inherent measures required for a disease "marker":

> The invention provides methods for characterizing an apparently healthy individual's risk of, and/or developing their risk profile for developing a future subject cardiovascular disorder. The method comprises obtaining a level of MIF in the individual, typically expressed as MIF concentration, and comparing the level of the marker to a predetermined value. The individual's risk or risk profile of developing a future subject cardiovascular disorder then is characterized based upon the level of the marker in comparison to the predetermined value. Specification, p.3, lines 14-20.

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The recited predetermined value is a control:

> The predetermined value will depend upon the characteristics of the patient, and/or the relevant patient population. The predetermined value can be a single value, multiple values, a single range or multiple ranges. Thus, in one embodiment, the predetermined value is a plurality of predetermined marker level ranges, and the comparing step comprises determining in which of the predetermined marker level ranges the individual's level falls. In another embodiment, the predetermined value is a historical value from the patient. Specification, p.4, lines 11-16.

The Specification even expressly refers to the compared-to or "predetermined value" a "control":

> I. Comparison of MIF and CRP levels as correlates to reductions in cardiovascular risk. This study was designed to compare MIF and CRP as markers correlating with cardiovascular risk.
> Methods: In an initial demonstration, we monitored MIF in obese adults, with very high cardiovascular risk, who were subjected to a one-year regimen of diet and exercise.
> Results: We found that MIF levels tracked progress (reduction in cardiovascular risk) through the treatment regimen better than did CRP. In our ***control*** group (n=83), MIF levels were 38 +/-16 ng/ml. The obese patients at baseline are elevated to 100+ ng/ml generally and drop to normal levels generally after 1 year. Specification, p.5, line 7 (emphasis added)

In my opinion, that the determined MIF concentration is a "test", and the compared-to value is a "control" is both self-evident and inherent in the original claims. In my opinion, the Specification plainly conveys possession of the invention as claimed to those skilled in the art.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application and any patent issuing therefrom.

Date: 1/2/07

Brett Giroir, M.D.

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Macrophage migration inhibitory factor is a cardiac-derived myocardial depressant factor

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Garner, Leslie B., Monte S. Willis, Deborah L. Carlson, J. Michael DiMaio, Michael D. White, D. Jean White, Glenn A. Adams IV, Jureta W. Horton, and Brett P. Giroir. Macrophage migration inhibitory factor is a cardiac-derived myocardial depressant factor. *Am J Physiol Heart Circ Physiol* 285: H2500–H2509, 2003. First published August 28, 2003; 10.1152/ajpheart.00432.2003.—Macrophage migration inhibitory factor (MIF) is a pluripotent proinflammatory cytokine that is ubiquitously expressed in organs, including the heart. However, no specific role for MIF in modulating cardiac performance has yet been described. Therefore, we examined cardiac MIF expression in mice after LPS challenge (4 mg/kg) and tested the hypothesis that MIF is a mediator of LPS-induced cardiac dysfunction. Western blots of whole heart lysates, as well as immunohistochemistry, documented constitutive MIF protein expression in the heart. Cardiac MIF protein levels significantly decreased after LPS challenge, reaching a nadir at 12 h, and then returned to baseline by 24 h. This pattern was consistent with MIF release from cytoplasmic stores after endotoxin challenge. After release of protein, MIF mRNA levels increased 24–48 h postchallenge. To determine the functional consequences of MIF release, we treated LPS-challenged mice with anti-MIF neutralizing antibodies or isotype control antibodies. Anti-MIF-treated animals had significantly improved cardiac function, as evidenced by a significant improvement in left ventricular (LV) fractional shortening percentage at 8, 12, 24, and 48 h after endotoxin challenge. In support of these findings, perfusion of isolated beating mouse hearts (Langendorff preparation) with recombinant MIF (20 ng/ml) led to a significant decrease in both systolic and diastolic performance [LV pressure (LVP), positive and negative first derivative of LVP with respect to time, and rate of LVP rise at developed pressure of 40 mmHg]. This study demonstrates that MIF mediates LPS-induced cardiac dysfunction and suggests that MIF should be considered a pharmacological target for the treatment of cardiac dysfunction in sepsis and potentially other cardiac diseases.

cardiac dysfunction; innate immunity; sepsis; lipopolysaccharide

MACROPHAGE MIGRATION INHIBITORY FACTOR (MIF) is a pluripotent cytokine whose mechanisms of action remain elusive despite nearly four decades of study. Although crystallized as a trimer, its physiologically relevant oligomerization state remains unclear, its putative membrane receptor is unknown, and the physiological relevance of its intracellular enzymatic activity as a tautomerase and oxidoreductase remains uncertain. Despite our lack of a precise mechanistic understanding, many studies have demonstrated that MIF has an important role in such diverse diseases as rheumatoid arthritis, delayed-type hypersensitivity, inflammatory lung disease, cancer, myocardial infarction, and, perhaps most importantly, septic shock (26). During septic shock, MIF is increased in the plasma of animals and humans, and the blockade of MIF activity by monoclonal or polyclonal antibodies results in a marked improvement in the survival of animals with experimentally induced sepsis (7, 11). However, the pathophysiological mechanism(s) for this survival benefit remains uncertain.

We have investigated the possibility that MIF is an inducer of myocardial dysfunction that is known to contribute significantly to the morbidity and mortality of sepsis in humans (12, 22). In both human patients and animal models, sepsis-associated cardiac dysfunction is characterized by biventricular dilatation, decreased systolic contractility, and diminished diastolic relaxation (31, 34, 41). Most available data suggest that its pathogenesis is multifactorial with systemic and myocardial-derived cytokines such as TNF-α being necessary and sufficient to induce its onset (8, 21, 22).

In addition to TNF-α, we sought to identify other cardiac-derived proteins that might mediate, by paracrine or autocrine mechanisms, myocardial dysfunction in sepsis and potentially other cardiac diseases. Screening microarray analysis of cardiac gene expression in mice suggested that MIF was expressed in the heart and was differentially regulated by LPS (unpub-

*L. B. Garner and M. S. Willis contributed equally to this work.

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AJP-Heart and Circulatory Physiology

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lished data). Moreover, MIF has been shown to be constitutively expressed in the heart and upregulated in a model of acute myocardial infarction in rats (45). Given the data that MIF inhibition improves outcome in animals with experimental sepsis and the fact that MIF can be upregulated in the heart experimentally, we designed these studies to determine the expression pattern of MIF in cardiomyocytes in vivo, to examine whether this expression was altered by LPS challenge, and, most importantly, to evaluate MIF's potential physiological effect on cardiac function.

MATERIALS AND METHODS

Antibodies and cytokines. Polyclonal goat anti-human MIF IgG and recombinant human MIF (rMIF; R&D Systems, Minneapolis, MN) were reconstituted in PBS and 0.1% BSA in PBS, respectively, measured into aliquots, and stored at −20°C until use. This polyclonal goat anti-human MIF antibody has been shown to cross-react with murine MIF and was used in Western blot experiments (3). A polyclonal rabbit anti-goat IgG-horseradish peroxidase (HRP) (Bio-Rad, Hercules, CA) used as a secondary antibody for Western blots was stored at 4°C until use. A polyclonal rabbit anti-rat MIF IgG (Torrey Pines BioLabs, Houston, TX) was used for immunohistochemistry and was shown to cross-react with murine MIF in Western blot experiments in our laboratory (data not shown). Two monoclonal mouse anti-mouse MIF IgG1 antibodies (XIV.15.5 and III.D.9, gift from Cytokine PharmaSciences) and a monoclonal mouse IgG_1 isotype control antibody (HB-49, gift from Cytokine PharmaSciences) were used in the echocardiographic studies. These antibodies were raised after immunization with both mouse and human MIF and recognize both human and mouse MIF (personal communication, Dr. V. de la Cruz, Cytokine PharmaSciences). Previous studies have demonstrated in vivo neutralization of MIF activity by both the XIV.15.5 antibody (15, 29) and III.D.9 antibody (6, 13, 24).

Animals and experimental design. C57BL/6J mice (Jackson Labs, Bar Harbor, ME) and C3H/HeJ mice (Harlan, Indianapolis, IN) were obtained at 6–10 wk of age and maintained in a specific pathogen-free environment. Commercial chow and tap water were made available ad libitum. All animal protocols were reviewed and approved by the University of Texas Southwestern Medical Center Institutional Animal Care Advisory Committee and were in compliance with the rules governing animal use as published by the National Institutes of Health. C57BL/6J mice were injected intraperitoneally with 4 mg/kg *Escherichia coli* 0111:B4 LPS (Sigma-Aldrich, St. Louis, MO) and killed postinjection at time points indicated in the figures by CO_2 asphyxiation and subsequent cervical dislocation. Uninjected mice were used as controls. Both anti-MIF antibodies (III.D.9 and XIV.15.5) and an isotypic control (HB-49) were injected (100 μg in 200 μl PBS) intraperitoneally 90 min before the LPS challenge in the echocardiogram studies. Whole hearts were removed, snap frozen in liquid nitrogen, and stored at −80°C or fixed in 10% neutral-buffered formalin for 24 h and placed in 70% ethanol for immunohistochemistry. Whole blood was collected by retro-orbital bleeding using a small-diameter heparin-coated (to prevent local clotting in the capillary tube) microhematocrit capillary tube (Allegiance Healthcare, McGaw Park, IL), which immediately directed the blood from the eye into a serum separator tube containing clot activators (Vacutainer SST Gel & Clot activator tubes, Becton Dickinson, Franklin Lakes, NJ). The tube was inverted five times, allowed to clot for a minimum of 30 min, and centrifuged at 1,200 *g* for 10 min at 4°C. Care was taken to avoid hemolysis during collection of serum. Serum was transferred to a sterile snap-top tube and frozen at −80°C until assayed by ELISA.

Protein extraction and Western blotting. Hearts were thawed and homogenized on ice in Tris-buffered saline (TBS, 50 mM Tris and 150 mM NaCl, pH 7.5) containing 1% NP40, 0.5% deoxycholic acid, 0.1% SDS, 2 mM EDTA, and 1 mM PMSF. Lysate protein concentration was quantified using the Bio-Rad Protein Assay. Protein (20 μg) was diluted 2 parts sample with 1 part Laemmli sample buffer (Bio-Rad) and resolved on an 18% SDS polyacrylamide gel under reducing conditions. Prestained SDS-PAGE standards (Kaleidoscope Broad range, Bio-Rad Laboratories) were run with each gel to determine the approximate molecular weight of the detected bands. Additionally, rMIF (R&D Systems) was used as a positive control on initial Western blots. The gel was transferred to polyvinylidene difluoride membranes (NEN, Boston, MA) using a semidry transfer apparatus (Bio-Rad) at 15 V for 15 min. The membrane was then blocked with TBS-0.1% Tween 20 (TBS T) with 0.5% nonfat dry milk (Bio-Rad) for 30 min and incubated with polyclonal goat anti-human MIF IgG (1:750) in TBS-T with 0.5% nonfat milk overnight at 4°C. The membrane was washed three times for 10 min in TBS-T, incubated with rabbit anti-goat IgG-HRP (1:1,000) for 1 h at room temperature, and washed four times for 10 min with TBS-T. The membrane was then exposed to 5 ml of a mixture of luminol plus hydrogen peroxide under alkaline conditions (SuperSignal West Pico, Pierce, Rockford, IL) for 5 min, and the resulting chemiluminescent reaction was detected by Kodak X-OMAT AR Film (Eastman Kodak, Rochester, NY).

The quantification of the single band density with the approximate molecular mass of MIF (12.5 kDa) was determined using Quantity One software (Bio-Rad, version 4.4.0, build 36) after conversion of radiographic film to TIFF files (8-bit grayscale) using a Scanjet 7400c (Hewlett-Packard, Palo Alto, CA) and reported in arbitrary units (AU) per square millimeter. Densitometry was performed by outlining the MIF bands with the volume rectangle tool initially set on the control band of interest. This rectangle was then copied and pasted onto other bands that were completely outlined, and the volume analysis report was run.

RNA extraction, probe preparation, and Northern blotting. Hearts were thawed on ice, and total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and quantified by spectrophotometry. An MIF-specific Northern probe was prepared by isolating DNA (DNeasy Tissue Kit, Qiagen, Valencia, CA) from an MIF plasmid (Research Genetics, Huntsville, AL) and subsequently cutting it with *Eco*RI and *Not*I restriction enzymes (Fisher Scientific, Pittsburgh, PA). The resultant DNA was resolved on a 1.2% agarose gel, purified (GenElute Agarose Spin Columns, Supelco, Bellefonte, PA), labeled with 5 μl [^{32}P]dCTP (3,000 Ci/mmol) (Perkin-Elmer, Boston, MA) using Ready-To-Go Labeling Beads (Amersham Pharmacia, Piscataway, NJ), and purified in ProbeQuant Microcolumns (Amersham Pharmacia) according to manufacturers' protocols.

RNA (10 μg) was resolved on 1.2% agarose gels at 100 V for 1 h and transferred to a Hybond-N$^+$ membrane (Amersham Pharmacia, Buckingham, UK) at 1.5 A for 1 h on a transfer electrophoresis unit (TransPhor PowerLid, Hoefer Scientific Instruments, San Francisco, CA). RNA was linked to the membrane for ~2 min using a GS Gene Linker set at C3 (Bio-Rad). The membrane was prehybridized in a hybridization oven (Sorvall Life Science, Greensboro, NC) in Perfect-

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Hyb Plus (Sigma, St. Louis, MO) for 1 h at 68°C. Sheared, denatured salmon testis DNA (100 μg/ml) was then added for 1 h, followed by the addition of ~0.1 μg probe labeled at >5 × 10^8 counts·min^{-1}·μg^{-1}. The blot was then hybridized for 12 h at 68°C in the hybridization oven followed by washing at 68°C in 2× standard saline citrate and 0.1% SDS. The membrane was washed for 1 h, the buffer was exchanged, and then the membrane was washed for an additional 1 h at 68°C. The membrane was wrapped in Saran Wrap, and mRNA was detected by Kodak X-OMAT AR film after 96 h (Eastman Kodak). The same membrane was stripped and then reprobed in a similar manner with radiolabeled β-actin (0.1 μg probe labeled at >5 × 10^8 counts·min^{-1}·μg^{-1}) (Ambion, Austin, TX). Densitometry was performed as described above for the Western blots. The β-actin mRNA bands served as a control against which to normalize the MIF mRNA densitometry.

Immunohistochemistry. Tissue was fixed in neutral buffered formalin, processed to paraffin, and subsequently immunostained at room temperature on a BioTek Solutions Techmate 1,000 automated immunostainer (Ventana Medical Systems, Tucson, AZ) using the Ultra-streptavidin biotin system with HRP and diaminobenzidine (DAB) chromogen (Signet Laboratories, Dedham, MA). Optimum primary antibody concentrations were predetermined using known positive control tissues (LPS-challenged rat as previously described) (4). Paraffin sections were cut at 3 μm on a rotary microtome, mounted on positively charged glass slides (POP100 capillary gap slides, Ventana Medical Systems), and air-dried overnight. Sections were then deparaffinized in xylene and ethanol, quenched with fresh 3% hydrogen peroxide for 10 min to inhibit endogenous tissue peroxidase activity, and rinsed with deionized water. Sections were incubated in unlabeled blocking serum for 15 min to block nonspecific binding of the secondary antibody and then incubated for 25 min with either the polyclonal rabbit anti-rat MIF IgG (1:400, Torrey Pines BioLabs, Houston, TX) diluted in 1% citrate buffer (BioPath, Oklahoma City, OK) or with buffer alone as a negative reagent control. A negative reagent control was run for each time point and for each organ. After washes in buffer, sections were incubated for 25 min with a biotinylated polyvalent secondary antibody solution (containing goat anti-rabbit IgG). Next, sections were washed with buffer, incubated in HRP-conjugated streptavidin-biotin complex for 15 min, washed again in buffer, and then incubated with two changes, 5 min each, of a freshly prepared mixture of DAB and H_2O_2 in buffer, followed by washing in buffer and then water. Sections were then counterstained with hematoxylin, dehydrated in a graded series of ethanol and xylene, and placed under coverslips. Slides were reviewed by light microscopy, and positive reactions with DAB were identified as a dark brown reaction product.

Determination of cardiac function in response to recombinant MIF. C57BL/6J and C3H/HeJ mice were used in the Langendorff assays as previously described (44). Briefly, 200 units of heparin sulfate were given intraperitoneally, the mice were killed 20 min later, and the heart was immediately removed and placed on ice in Krebs-Henseleit buffer [in mM: 2 $NaHCO_3$, 118 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 11.1 glucose, pH 7.4, which was prepared fresh with demineralized, deionized water and bubbled with 95% O_2-5% CO_2 (Po_2 590 mmHg, Pco_2 38 mmHg)]. The aorta was cannulated with PE-50 tubing, and the heart was perfused in a retrograde manner through the aortic root with prefiltered, oxygenated Krebs-Henseleit buffer at a constant flow rate of 1.5 ml/min (temperature 37°C) and a recirculating volume of 100 ml. The heart was placed in a water-jacketed chamber to maintain constant temperature and humidity. PE-50 intramedic polyethylene tubing, connected to a Statham pressure transducer, was inserted into the left ventricle (LV) to measure LV pressure (LVP). Temperature was monitored using a 27-gauge thermistor needle inserted into the LV muscle. After instrumentation, hearts were allowed to stabilize for 10 min, and hearts that failed to achieve a stable pressure or developed persistent arrhythmias during this time were excluded from the study. After stabilization, LVP and its first derivative (dP/dt), heart rate, and coronary perfusion were measured simultaneously with a multichannel Grass 7D polygraph (Grass Instruments, Quincy, MA). Ventricular performance as a function of coronary perfusion was determined for all hearts by plotting peak systolic LVP and ±dP/dt_{max} values against incremental increases in coronary flow rate. Hearts were perfused with or without 20 ng/ml rMIF added to the perfusate for 20 min before readings were taken.

Determination of cardiac dysfunction by echocardiography. Echocardiograms to assess systolic function were performed using M-mode measurements. Mice were anesthetized with 5% isoflurane with 2.5 l/m O_2 for 20 s (until unconscious) followed by 2% isoflurane and O_2 for an average of 12–15 min. Hair was removed from the thorax and upper abdomen using Nair hair remover and gauze after sitting for 3 min. Echocardiographic measurements were obtained on anesthetized mice ~5–8 min after induction to allow any transient anesthesia-related cardiac depression to resolve. Transient, minimal changes in cardiac function detected by echocardiography have been reported after inhaled isoflurane, although fractional shortening percentage (FS%) has been reported to be stable (39). Echocardiography was performed using a Hewlett-Packard Sonos 5500 (Agilent Technologies; Edmonton, Alberta, Canada) with a frame rate of 300–500 frames/s in a random and blinded manner. A 12-MHz linear transducer was placed on the left hemithorax interfaced with a layer of US transmission gel (Aquasonic 100, Parker Laboratories; Fairfield, NJ). The two-dimensional parasternal short-axis imaging plane guided LV M-mode tracings close to the papillary muscle level. Depth was set at a minimum of 2 cm with a sweep speed of 150 m/s. Tracings were printed on a Sony color printer (UP-5200, Sony).

M-mode measurements. Data represent the average of nine selected cardiac cycles from at least two separate scans. End diastole was defined as the maximal LV diastolic dimension, and end systole was defined as the peak of posterior wall motion. FS%, a surrogate of systolic function, was calculated from LV dimensions as follows: FS% = (LVED − LVES)/LVED × 100, as shown in Fig. 5*A*, where LVED and LVES are LV dimensions at end diastole and end systole, respectively.

Determination of serum MIF levels. Sera from six mice per time point were assayed for mouse MIF using the Chemikine rat/mouse macrophage inhibitory factor (MIF) EIA kit (Chemicon International, Temecula, CA) according to the manufacturer's instructions. Briefly, 5 μl of standards, samples, or reaction buffer (blank) were added to each well in triplicate. Next, 100 μl of diluted MIF-HRP antibody conjugate were added to each well and allowed to incubate for 2 h at room temperature. Wells were then washed five times, and 100 μl of 3,3′,5,5′-tetramethylbenzidene substrate were added and allowed to incubate in the dark for 30 min at room temperature. The stop reagent (0.5 N H_2SO_4-0.5 HCl) was added to each well and gently mixed, and the ELISA was read on an MRX Revelation microtiter plate reader (Dynex Technologies, Chantilly, VA) at 450 nm within 30 min of completion of the assay (reference at 630 nm).

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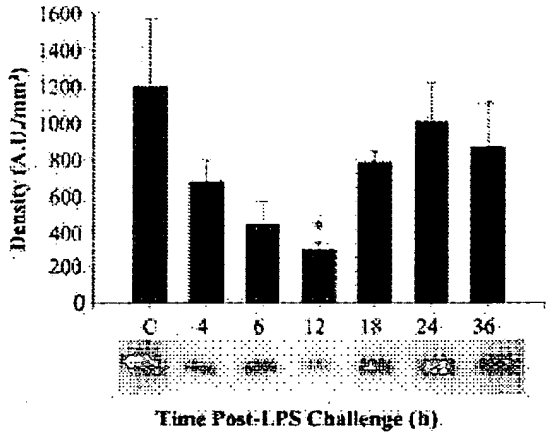

Fig. 1. Macrophage migration inhibitory factor (MIF) is constitutively expressed in cardiac tissue and released maximally 12 h post-LPS challenge. Each data point represents the mean density in arbitrary units (AU)/mm² ± SE of 3 independent Western blot experiments. A representative Western blot is shown below the graph. One-way ANOVA and multiple-comparison procedure using the Tukey method were employed to determine statistical significance compared with the control group (*$P < 0.05$). C, control.

Statistical analysis. Northern and Western data are expressed as means ± SE and were statistically analyzed using a one-way ANOVA. A multiple-comparison procedure was employed using the Tukey method to determine statistical significance between groups. Cardiac function determined by the Langendorff preparation (including stabilization data) is expressed as the mean ± SE, and separate analyses were performed for each parameter measured (e.g., LVP, $+dP/dt_{max}$) as a function of treatment group and coronary flow rate using a repeated-measures ANOVA. A multiple comparison procedure employing the Bonferroni method was used to determine significant differences between groups. Serum MIF levels are expressed as means ± SE and were statistically analyzed using a one-way ANOVA, with a multiple-comparison procedure employing the Bonferroni method to determine significance between groups. Cardiac function determined by M-mode echocardiography is expressed as FS% ± SE and analyzed using a one-way repeated-measures ANOVA. Additional comparisons were performed using the Tukey test to determine significant differences between specific groups. Statistical significance for all analyses was defined as $P < 0.05$. All statistical analyses were performed using SigmaStat 2.03 (SPSS, Chicago, IL) and Microsoft Excel (Microsoft, Seattle, WA).

RESULTS

MIF protein is constitutively expressed by cardiac myocytes in vivo and is released in response to LPS challenge. Both immunochemistry and Western analysis performed on cardiac tissue documented the presence of MIF in cardiac cells, including ventricular and atrial myocytes, under baseline control conditions (Figs. 1 and 2). After endotoxin challenge, both immu-

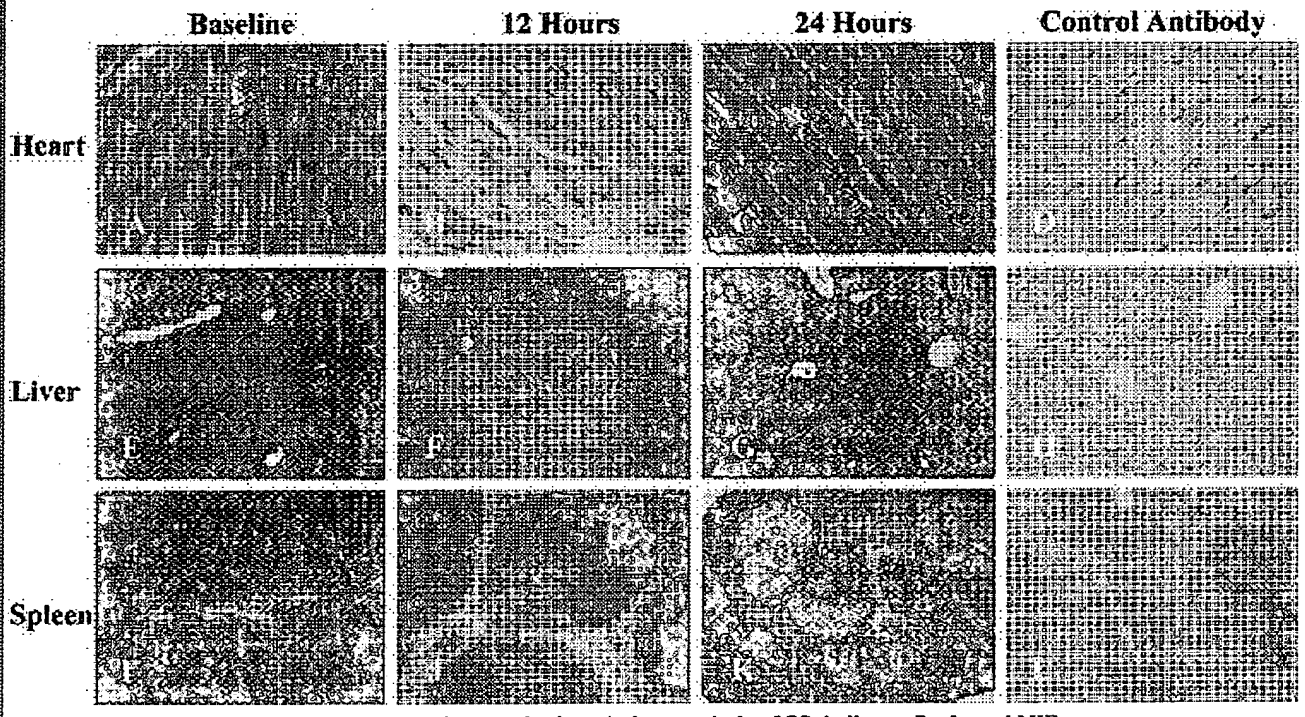

Fig. 2. MIF, constitutively present in the heart, liver, and spleen, is decreased after LPS challenge. Preformed MIF in the heart, liver, and spleen (*A*, *E*, *I*) decreases 12 h after LPS challenge (*B*, *F*, and *J*) and is replenished after 24 h (*C*, *G*, and *K*) as demonstrated by immunohistochemistry. A negative control (secondary antibody without the primary anti-MIF antibody) demonstrated that no background staining was present at each time point in each organ investigated as represented in the far right column (*D*, *H*, *L*). Magnification: ×100 (kidney, spleen); ×400 (heart).

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Table 1. *Serum MIF concentration after a 4 mg/kg endotoxin challenge*

Baseline	4 h	8 h	12 h	24 h	48 h
79.1±4.6	90.9±8.7	118.1±5.6*	81.4±5.8	70.1±5.1	69.9±9.0

Data are in ng/ml and are expressed as means ± SE of 6 C57BL/6J mice as determined by ELISA and were statistically analyzed using a 1-way ANOVA with a multiple-comparison procedure, employing the Bonferroni method to determine significance between groups (*$P < 0.05$ compared with baseline). MIF, macrophage migration inhibitory factor.

nochemistry and immunoblot analysis document a significant decrease in cardiac tissue MIF concentration. This decrease was most profound (75% decrease) at 12 h with levels returning to near baseline control levels by 24 h. This expression pattern in the heart is similar to that witnessed in the liver and spleen (Fig. 2) and is consistent with the hypothesis that MIF is released from preformed stores within tissue after LPS challenge. The release of MIF from tissue evident at 4 h on immunoblot (Fig. 1) correlates with the increase in serum levels after endotoxin exposure (Table 1).

Myocardial MIF mRNA expression increases after endotoxin challenge. Northern blot analysis of RNA obtained from the hearts of either control or LPS-challenged mice at given time points indicates that MIF mRNA is constitutively expressed in the hearts of control mice and that significant increases in MIF mRNA concentration are detectable at 48 h after LPS challenge (Fig. 3).

MIF induces systolic and diastolic cardiac dysfunction in an LPS-independent mechanism. To determine if MIF directly influences cardiac function, spontaneously beating normal mouse hearts (Langendorff preparation) were perfused with rMIF at a concentration of 20 ng/ml, approximating that documented in the serum of humans with septic shock (18). The human MIF used in the Langendorff perfusion studies has been shown to have an ~90% homology with murine MIF (30) and has been shown to have cross-species biological function (5, 30). Therefore, its activity should be similar to murine MIF. Responses to MIF were determined in hearts from both C57BL/6J and C3H/HeJ mice. C3H/HeJ mice are resistant to endotoxin (35–37), thereby controlling for the possibility that any depression observed might be due to trace amount of endotoxin in the perfusate. Table 2 illustrates the responses of both mouse strains to retrograde aortic perfusion at 1.5 ml/min with control perfusate or perfusate containing 20 ng/ml rMIF after stabilization. Perfusion with MIF led to a significant decrease in LVP, $+dP/dt_{max}$, $-dP/dt_{max}$, and rate of LVP rise at a developed pressure of 40 mmHg (dP40; mmHg/s) in both mouse strains, whereas other parameters (time to maximal $\pm dP/dt$, coronary perfusion pressure, coronary vascular resistance, and heart rate) were unaffected. Figure 4 illustrates the effect of rMIF over a range of coronary flow rates. Increases in coronary flow produced a stepwise increase in contractile performance in all hearts regardless of experimental group assignment. Comparison of the rMIF-challenged hearts with control hearts revealed a downward shift in the function curves, indicating significant systolic and diastolic depression in response to 20 ng/ml rMIF ($P < 0.05$). The effect of rMIF was statistically identical in both endotoxin-sensitive (C57BL/6J) and endotoxin-resistant (C3H/HeJ) strains. Likewise, there were no differences in LVP, $+dP/dt_{max}$, and $-dP/dt_{max}$ between the C57BL/6J and C3H/HeJ study hearts perfused with rMIF.

Anti-MIF antibodies improve LPS-induced cardiac depression in vivo. To determine the influence of MIF in the pathogenesis of cardiac dysfunction in vivo, serial echocardiography (M-mode) was performed on LPS-challenged mice that had been pretreated (90 min prior) with either of two anti-MIF monoclonal antibodies, an isotype control antibody, or no treatment (Fig. 5). At 4 h post-LPS challenge, the FS% of all LPS-challenged mice was similarly depressed (50% reduction in FS%), irrespective of group assignment. Eight hours post-LPS challenge, however, mice injected with either of the two monoclonal anti-MIF antibodies demonstrated statistically significant recovery of FS% compared with LPS-challenged groups receiving either no treatment or isotype antibody control (Fig. 5). This enhanced recovery of function persisted at 12, 24, and 48 h. At 48 h after challenge, anti-MIF-treated groups had near total restoration of FS%, whereas LPS-challenged groups receiving isotype control or no pretreatment remained profoundly depressed. Throughout the 48 h, the FS% of sham-operated mice did not change significantly, indicating that cardiac function was unaffected by anesthesia or the testing regimen itself. Additionally, at all time points, the mice injected with isotypic antibody controls were identical to animals

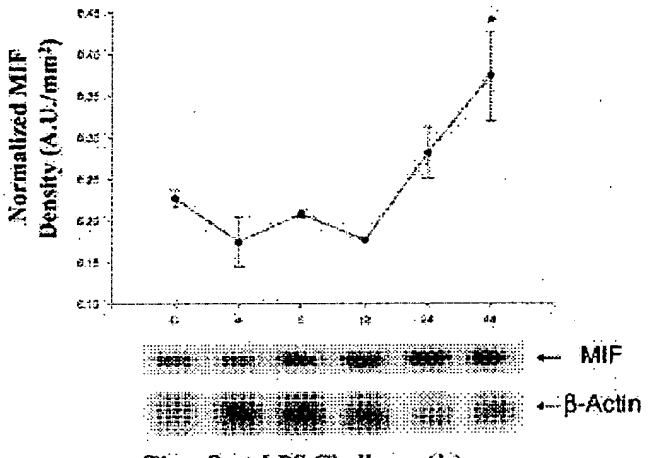

Fig. 3. LPS challenge upregulates MIF mRNA in cardiac tissue significantly at 48 h. MIF and β-actin mRNAs were detected using ^{32}P-radiolabeled probes complementary to MIF and β-actin mRNAs. Each data point represents the mean density in AU/mm² ± SE of 3 independent Northern blot experiments. A representative Northern blot is shown below the graph. Normalized MIF was determined by the ratio of MIF density to β-actin density. One-way ANOVA and multiple-comparison procedure using the Tukey method were employed to determine statistical significance compared with the control group (*$P < 0.05$).

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Table 2. *In vitro stabilization data from isolated hearts in the Langendorff perfusion experiments*

Cardiac Function Tested	C57BL/6J Control ($n = 10$)	C57BL/6J Recombinant human MIF ($n = 10$)	C3H/HeJ Control ($n = 7$)	C3H/HeJ Recombinant human MIF ($n = 7$)
LVP, mmHg	92.9 ± 2.3	77.9 ± 5.1*	93.7 ± 3.5	75.2 ± 5.5*
+dP/d*t*, mmHg/s	2,180 ± 60	1,920 ± 89*	2,250 ± 42	1,800 ± 106*
−dP/d*t*, mmHg/s	1,856 ± 65	1,545 ± 122*	1,809 ± 84	1,343 ± 138*
dP40, mmHg/s	1,890 ± 57	1,655 ± 91*	1,914 ± 51	1,571 ± 101*
Time to maximum −dP/d*t*, ms	51.6 ± 2.3	48.3 ± 2.0	52.1 ± 2.1	47.6 ± 1.4
Time to maximum −dP/d*t*, ms	51.6 ± 1.0	50.6 ± 1.1	51.9 ± 1.6	52.0 ± 1.5
CPP, mmHg	73.0 ± 7.3	76.2 ± 7.7	86.0 ± 9.4	80.0 ± 9.6
CVR, mmHg	48.9 ± 15.4	50.9 ± 5.1	57.4 ± 6.3	53.4 ± 6.4
HR, beats/min	363 ± 16	339 ± 15	357 ± 19	338 ± 25

Cardiac function values are expressed as means ± SE. Separate analyses were performed for each parameter (left column) as a function of treatment group and coronary flow rate. LVP, left ventricular pressure; ±dP/d*t*, positive and negative 1st derivative of LVP with respect to time; dP40, rate of LVP rise at developed pressure of 40 mmHg; CPP, coronary perfusion pressure; CVR, coronary vascular resistance; HR, heart rate. A repeated-measures ANOVA with a multiple-comparison procedure employing the Bonferroni method was used to determine significant differences between groups (*$P < 0.05$ compared with control).

challenged with LPS alone, indicating specificity of the anti-MIF antibody effects.

DISCUSSION

This study is the first to demonstrate that macrophage MIF is a myocardial depressant factor and, in this regard, functions as an important late mediator of endotoxin-induced cardiac dysfunction in vivo. The timing of improved cardiac function associated with MIF blockade was consistent with the time course of MIF release from the myocardium and other tissues. Moreover, because the myocardium is itself a significant tissue source of MIF, these data raise the possibility that MIF may mediate cardiac dysfunction in other cardiac diseases.

As demonstrated in our echocardiography studies, MIF neutralization using anti-MIF antibodies confers significant protection from LPS-induced cardiac dys-

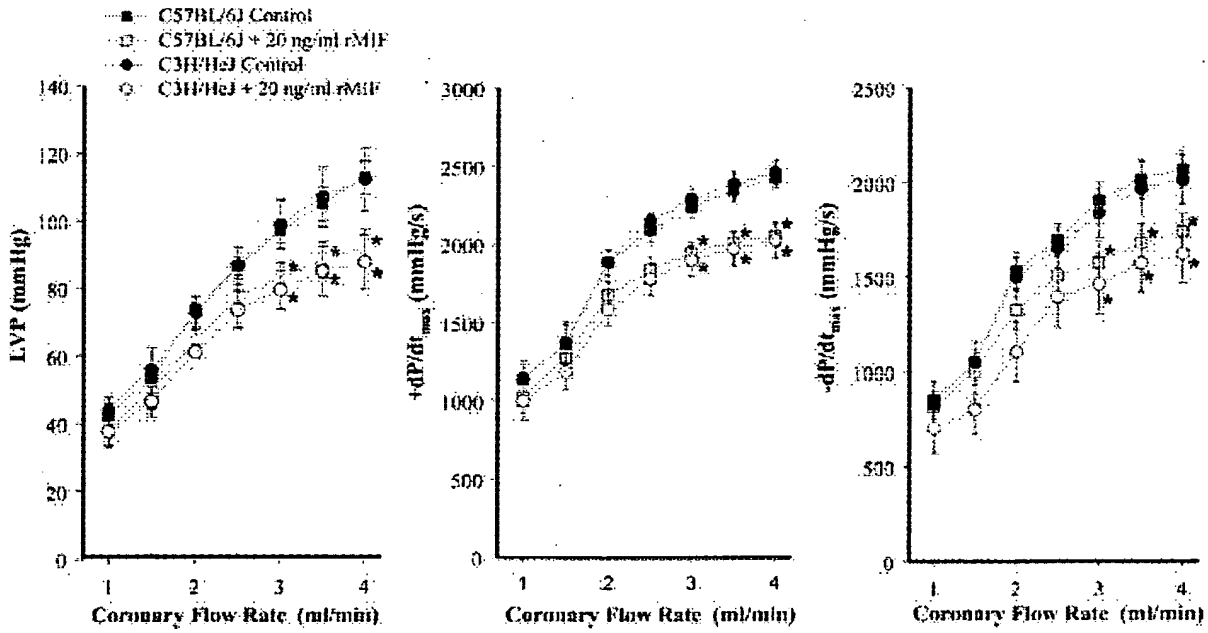

Fig. 4. Cardiac function determination by Langendorff preparation post-recombinant human MIF (rMIF) perfusion in C57BL/6J mice and endotoxin-resistant C3H/HeJ mice demonstrates that rMIF mediates cardiac dysfunction in an LPS-independent mechanism. Cardiac function is expressed as means ± SE of 7 C3H/HeJ and 10 C57BL/6J independent Langendorff experiments. Separate analyses were performed for each left ventricular (LV) pressure (LVP), maximum positive and negative first derivative of LVP with respect to time (+dP/dt_{max} and −dP/dt_{max}, respectively) as a function of treatment group and coronary flow rate using a repeated-measures ANOVA and multiple-comparison procedure employing the Bonferroni method to determine significant differences between groups (*$P < 0.05$).

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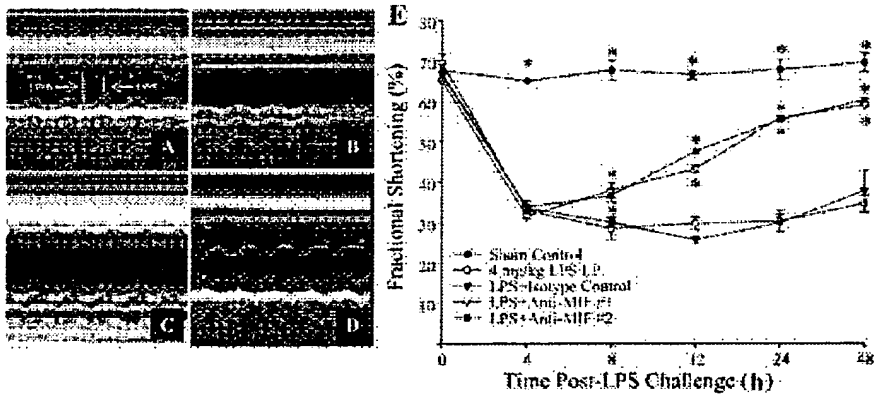

Fig. 5. Echocardiographic assessment of the effects of anti-MIF antibody therapy after LPS administration demonstrates the protective effects of MIF blockade. Representative M-mode echocardiograms in wild-type mice at baseline and 8 h after LPS administration (*A* and *B*, respectively). *C* and *D* depict representative echocardiograms in LPS plus anti-MIF treated mice at 8 and 48 h, respectively. A significant recovery of cardiac function [fractional shortening percentage (FS%)] is observed in LPS-challenged mice when anti-MIF antibodies are given pretreatment (*E*). Data from each group represent means ± SE of 9 cardiac cycles from 3 mice monitored at multiple time points. Cardiac function determined by echocardiography is expressed as FS% [(LVED − LVES)/LVED × 100] ± SE, where LVED and LVES are LV dimensions at end diastole and end systole, respectively, and was analyzed using a 1-way repeated-measures ANOVA and multiple-comparison procedure employing the Tukey test to determine significant differences between specific groups ($^*P < 0.05$), compared with the sham control group.

function beginning at 8 h post-LPS challenge (Fig. 5). Moreover, we have shown that rMIF induces nearly immediate cardiac depression ex vivo in the Langendorff perfusion studies (Fig. 4). Taken together, these data indicate that MIF must be locally present at the heart by 8 h after LPS challenge to mediate its cardiodepressant effects. Whether the source of this MIF is systemic or local is not entirely clear, but this study suggests that local MIF production may be important. The maximum circulating MIF levels after LPS challenge are modest, increasing only 1.5-fold by 8 h (Table 1); however, the maximum MIF release from cardiac tissue is more dramatic, decreasing fourfold by 12 h (Fig. 1). Because the timing and magnitude of MIF release from the heart itself is more impressive then systemic changes in MIF, local MIF release from the heart is more likely to be responsible for the cardiodepressant effects.

Further evidence that myocardial MIF works in an autocrine fashion to induce cardiac dysfunction after LPS challenge is demonstrated in the timing of maximal protection from LPS-induced cardiac dysfunction provided by MIF neutralization using anti-MIF antibodies (Fig. 5). Relatively minimal, although significant, recovery of heart function occurs at 8 h post-LPS challenge when circulating serum MIF levels are highest. However, maximal recovery of cardiac function is observed at 48 h post-LPS challenge when serum levels have returned to baseline. The time course for the recovery of heart function more closely correlates with the maximal release of MIF from the myocardium, although MIF's immediate effect in vitro is not likely the only effect expected as prolonged effects in vivo have been reported (28, 32). Similar local (autocrine) effects have been described with myocardial TNF-α production. Specifically, transgenic mice that overproduce TNF-α specifically in the heart develop a reproducible cardiomyopathy (20).

Previous studies have demonstrated a greater than five- to sixfold increase in serum MIF by ~2 h post-LPS challenge (9). In this study, both the maximum increase in serum MIF (1.5-fold of baseline vs. 5- to 6-fold previously reported) and the timing of MIF release into the serum (maximum at 8 h vs. 2 h previously reported) differ from previous reports. Possible reasons for this relatively blunted and slightly delayed response include strain differences among mice, the LPS dosage, and differences in assay sensitivities. Strain differences in MIF responses have been previously reported, specifically between C57BL/6J and BALB/c mice (1). Cells from BALB/c mice were shown to consistently secrete significantly higher levels of MIF in response to various stimuli compared with other stains (including C57BL/6J) (1). The MIF serum response may also differ because of the higher dose (4 mg/kg) used in this study compared with the previous report in BALB/c mice (9). Prior studies have demonstrated that increasing concentrations of LPS blunt MIF production by macrophages (9). The reason for the relatively high serum "background" MIF concentration determined by ELISA compared with previous determinations by Western blot is not clear but has been reported by other investigators using the same ELISA kit (45). Great care was taken to avoid obvious causes of falsely increased MIF levels (as reported by the manufacturer) by ELISA such as hemolysis. Indirect evidence supporting the proposal that our baseline MIF levels are falsely elevated comes from echocardiographic studies. When healthy wild-type (C57BL/6J) mice are given anti-MIF antibodies alone, no increase in function is noted by echocardiography (unpublished data). This suggests that baseline MIF levels are not very high

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and/or that circulating MIF alone may not be sufficient to cause myocardial depressant effects. Indeed, other cofactors such TNF-α or LPS may be necessary in order for MIF to mediate cardiac dysfunction in vivo.

Cardiac dysfunction during sepsis (12, 22) is associated with poor outcome in both humans (2, 40) and animals (7, 11). We and others (19, 21, 23) have previously demonstrated that sepsis- or burn-associated cardiac dysfunction is primarily due to circulating myocardial depressant factors, including TNF-α. However, because TNF-α is a sentinel, rapid-response cytokine and is gone from the circulation days or weeks before the resolution of myocardial dysfunction, we entertained the possibility that additional important myocardial depressant proteins might exist. When preliminary microarray data on cardiac gene expression highlighted that MIF is expressed in cardiac tissue (not published), we entertained the hypothesis that MIF itself might be a myocardial depressant protein. It has already been well established that MIF plasma levels are significantly elevated in patients with sepsis or systemic inflammatory response syndrome (18, 25) and that the kinetics of MIF release occur several hours after the initial cardiac dysfunction after LPS challenge is observed. These data supported the hypothesis that MIF could be a myocardial depressant protein that might account for late, prolonged cardiac depression during sepsis. Indeed, our results indicate that MIF perfusion directly depresses cardiac function in vitro; moreover, treatment with either of two independent monoclonal antibodies directed against MIF mitigates late myocardial depression in our model.

Studies utilizing live bacteria, either by direct intraperitoneal injection of *E. coli* or by cecal ligation and puncture (CLP), have previously demonstrated that MIF plasma and/or peritoneal fluid levels increase several hours postchallenge and that antibodies against MIF protected these mice from lethal bacterial peritonitis (11). Interestingly, mice were protected when the antibodies were given as late as 8 h after the onset of infection (11). In the present study, evidence supporting a delayed release was seen by Western blot and immunohistochemistry, which both demonstrated significant release of MIF from cardiac, liver, and spleen tissue 12 h after LPS challenge. Indirect support for this delayed MIF release is also evidenced by the delayed onset of cardiac protection beginning at 8 h post-LPS challenge and continuing thereafter. The delayed release of MIF after LPS challenge makes MIF an interesting potential therapeutic target.

MIF has a number of properties that make it unique among cytokines. MIF is released preformed from numerous cell types, including the lymphocytes, macrophages, and the anterior pituitary (5, 10, 15, 27). However, the list of sources of MIF continues to grow and includes other tissues such as the heart, lung, liver, adrenal, spleen, kidney, skin, muscle, thymus, skin, and testes (4, 16, 45). MIF has at least two catalytic activities that are distinct: tautomerase and oxidoreductase activity. To this end, pharmacological inhibitors of MIF tautomerase activity have been developed for the treatment of MIF-related diseases such as sepsis, acute respiratory distress syndrome, asthma, atopic dermatitis, rheumatoid arthritis, nephropathy, and cancer (14, 33). Most of these diseases have shown benefit from treatment with anti-MIF antibodies, at least in animal models; whether specific inhibition of tautomerase or oxidoreductase enzymatic activity might yield similar benefits remains untested.

We have not yet determined the precise mechanism by which MIF affects cardiac function. However, several investigations indicate that MIF may exert effects by both direct and indirect mechanisms. Previous studies have provided evidence that MIF promotes the release and pharmacodynamic effects of other proinflammatory cytokines. Macrophages expressing antisense MIF cDNA (leading to less endogenous MIF) secrete/express significantly less TNF-α, IL-6, and nitric oxide (NO), while NF-κB activity is decreased in response to LPS (38). Therefore, it appears that MIF may directly interact with the LPS signaling pathway by unknown mechanisms (26). Moreover, MIF knockout mice, which are resistant to lethal doses of LPS, have lower circulating plasma levels of TNF-α compared with wild-type mice at baseline. On LPS challenge, these mice demonstrate diminished circulating TNF-α concentrations, increased NO concentrations, and unchanged IL-6 and IL-12 concentrations (7). While MIF appears to promote proinflammatory cytokines, the effects of MIF have been shown to act in a TNF-α-independent manner in sepsis. When CLP was performed in TNF-α knockout mice, a 60% survival rate (at 15 h) was seen in mice administered anti-MIF antibodies compared with a 0% survival rate in TNF-α knockout mice (11).

In relation to cardiac dysfunction unrelated to sepsis, elevated serum MIF concentrations have been described in patients after acute myocardial infarction (42, 43, 46) with heretofore unknown physiological relevance. In addition, increased tissue expression of MIF occurs in the myocardium following a model of acute myocardial infarction in the rat (45). Similarly, cultured cardiac myocytes have been noted to release MIF in response to hypoxia and hydrogen peroxide (free radical initiator) but not angiotensin II, endothelin-1, IL-1β, or TNF-α (17, 43). Taken together, these results suggest that there are many clinical scenarios that could potentially trigger myocardial MIF release, thereby adversely affecting cardiac function, for which anti-MIF therapies may be of benefit.

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DISCLOSURES

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Systolic blood pressure response to exercise stress test and risk of stroke.

Kurl S, **Laukkanen JA**, **Rauramaa R**, **Lakka TA**, **Sivenius J**, **Salonen JT**.

Research Institute of Public Health, Department of Public Health and General Practice, University of Kuopio, Finland.

BACKGROUND AND PURPOSE: Systolic blood pressure (SBP) during exercise has been found to predict a future diagnosis of hypertension, coronary heart disease, and cardiovascular disease death. No studies have been conducted to show a relationship between SBP during exercise test and stroke. The aim of the present study was to study the associations between SBP rise, percent maximum SBP at 2 minutes after exercise, and the risk of stroke in a population-based sample of men with no prior coronary heart disease. METHODS: SBP was measured every 2 minutes during and after the exercise test. The subjects were a population-based sample of 1026 men without clinical coronary heart disease, antihypertensive medication, or prior stroke at baseline. During an average follow-up of 10.4 years, there were 46 cases of stroke (38 ischemic strokes). RESULTS: Men with SBP rise >19.7 mm Hg per minute of exercise duration had a 2.3-fold increased risk of any stroke and a 2.3-fold increased risk of ischemic stroke compared with men whose SBP rise was <16.1 mm Hg/min. Similarly, percent maximum SBP at 2 minutes after exercise (SBP at 2 minutes' recovery divided by maximum SBP) was associated (highest tertile) with a 4.6-fold increased risk of any stroke and a 5.2-fold increased risk of ischemic stroke. CONCLUSIONS: SBP rise during exercise and percent maximum SBP at 2 minutes after exercise were directly and independently associated with the risk of all stroke and ischemic stroke. Exercise SBP testing may be recommended as an additional tool in the prediction of future stroke.

PMID: 11546894 [PubMed - indexed for MEDLINE]

Related Links

- Stress-induced blood pressure reactivity and incident stroke in middle-aged men.
- Association of exercise-induced, silent ST-

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Effect of plasma C-reactive protein levels in modulating the risk of coronary heart disease associated with small, dense, low-density lipoproteins in men (The Quebec Cardiovascular Study).

St-Pierre AC, **Bergeron J**, **Pirro M**, **Cantin B**, **Dagenais GR**, **Despres JP**, **Lamarche B**; **Quebec Cardiovascular Study**.

Institute on Nutraceuticals and Fonctionnal Foods, Laval University, Ste-Foy, Quebec, Canada.

This purpose of this study was to investigate how plasma C-reactive protein (CRP), a nonspecific acute-phase reactant, modulates the risk of coronary heart disease (CHD) associated with the small, dense, low-density lipoprotein (LDL) phenotype. LDL particle size and plasma CRP were measured in the Quebec Cardiovascular Study cohort of 2,025 men free of CHD at baseline, among whom 103 had a first CHD event during a 5-year follow-up period. Plasma CRP levels were measured using the Behring Latex-Enhanced (highly sensitive) CRP assay. LDL particle size phenotype was characterized using 2% to 16% polyacrylamide gradient gel electrophoresis. There were weak but significant associations between plasma CRP levels and features of LDL size, such as the proportion of LDL with a diameter <255 A ($r = 0.09$, $p < 0.001$) and LDL peak particle size ($r = -0.09$, $p < 0.001$). Variations in plasma CRP levels modulated the risk of CHD associated with small LDL peak particle size (relative risk 4.3 vs 2.5 in men with high vs low plasma CRP levels, respectively) and with an elevated proportion of LDL <255 A (relative risk 6.6 vs 3.0). Thus, increased plasma CRP levels further elevate the risk of CHD associated with having small, dense LDL particles.

PMID: 12615259 [PubMed - indexed for MEDLINE]

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- A prospective, population-based study of low density lipoprotein particle size as a risk factor for ischemic heart disease in men.
- Lipoprotein-associated phospholipase A2, high-sensitivity C-reactive protein, and risk for incident coronary heart disease in middle-aged men and women in the Atherosclerosis Risk in Communities (ARIC) study.

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